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Molecular Cloning and Identification of a Receptor-Type Protein Tyrosine Phosphatase, IA-2, from Human Insulinoma

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ABSTRACT

A novel 3.6-kb cDNA, IA-2, with a 2,937-bp open reading frame was isolated from a human insulinoma subtraction library (ISL-153). The predicted amino acid sequence and *in vitro*-translated product of IA-2 cDNA revealed a 979-amino-acid protein with a pI value of 7.09 and a molecular mass of 105,847 daltons. The protein sequence is consistent with a signal peptide, an extracellular domain, a transmembrane region, and an intracellular domain. The extracellular domain contains an unusual cysteine-rich region following the signal peptide. The intracellular cytoplasmic domain of IA-2 possesses highly conserved regions similar to the catalytic domains found in members of the protein tyrosine phosphatase (PTP) family. Northern blot analysis showed that IA-2 mRNA was expressed in five of five freshly isolated human insulinomas, rat and mouse insulinoma cell lines, and enriched normal mouse islets. It also was found in normal human brain, pituitary, pancreas, and brain tumor cell lines, but not in a variety of other normal or tumor tissues. Based on the sequence and expression data, it appears that IA-2 is a new member of the receptor-type PTP family that is expressed in islet and brain tissues.

INTRODUCTION

BETA CELLS ARE THE ONLY CELLS IN THE BODY that are definitively known to produce insulin. Destruction of beta cells leads to insulin-dependent diabetes mellitus (IDDM). A variety of molecules are associated with the regulation, synthesis, and release of insulin. Not all of these molecules, however, have been identified nor has the possibility been excluded that other hormones, in addition to insulin, are produced by beta cells.

In recent years, molecules, such as amylin (Cooper *et al.*, 1989), glutamic acid decarboxylase (Baekkeskov *et al.*, 1990), and pancreastatin (Tatemoto *et al.*, 1986), have been isolated that are uniquely or differentially expressed in beta cells. The possibility that still other unidentified beta cell-produced molecules may have hormonal function, play a role in the long-term complications of IDDM, or be the target for autoimmune IDDM makes it important to identify these molecules.

Recently, we described a strategy for isolating genes uniquely or differentially expressed in human beta cells (Goto *et al.*, 1992). A human insulinoma cDNA library (ISL-153) was constructed by subtracting glucagonoma phagemid cDNA from insulinoma phagemid cDNA. From this subtraction library, consisting of 153 clones, we identified cDNA clones that hybridized differentially with end-labeled mRNAs isolated from insulinomas, glucagonomas, and HeLa cells. Clones that hybridized preferentially with end-labeled insulinoma mRNA were further screened by Northern analysis with a panel of tumor cell lines and normal tissues. Each clone with restricted tissue specificity was partially sequenced (approximately 200 bp) and compared with GenBank DNA database.

Clones with restricted tissue specificity and novel sequences were studied in depth. In the present paper, we describe the isolation and expression of a full-length sequence of a novel cDNA, designated IA-2. IA-2 mRNA was found in all human, mouse, and rat insulinomas examined and also in normal islet and brain tissues. IA-2 encodes a protein of 979 amino acids with a highly conserved intracellular cytoplasmic region that resembles the catalytic domain of protein tyrosine phosphatase (PTP).

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MATERIALS AND METHODS

Human tissues and cell lines

Human insulinoma and glucagonoma tissues were obtained from the National Cancer Institute, National Institutes of Health (Bethesda, MD). Tissue type was confirmed by immunoperoxidase staining for insulin, glucagon, chromograinin, somatostatin, gastrin, synaptophysin, and pancreatic polypeptide. All five insulinomas stained positive for insulin and negative for glucagon. Normal human tissues were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Cell lines: HPAF-2, BT-20, SKMEL, DM-6, HeLa, and LS-180 were obtained from Dr. R.S. Metzgar (Duke University, NC); JAR, SK-N-SH, U-87-MG, PC-3, and SW-579 were obtained from the American Type Culture Collection (Rockville, MD). βTC-1 was kindly provided by Dr. E.H. Leiter (Bar Harbor, ME). Rat insulinoma cell line, RIN (Gazdar *et al.*, 1980), was propagated routinely in our laboratory. Tumor cell lines were cultured in modified Eagle's medium supplemented with 10% fetal calf serum or according to the supplier's instructions.

Isolation of IA-2 cDNA clones

A human insulinoma subtraction library (ISL-153) was constructed from an insulinoma phagemid cDNA library and a glucagonoma phagemid cDNA library as described earlier (Goto *et al.*, 1992). A clone, IA-2-134, extending 749 bp upstream from the poly(A) tail was selected for further study. To obtain the full-length IA-2 sequence, we constructed another random-primed λZAPII (Stratagene, La Jolla, CA) human insulinoma library (RP-IL) and screened that library with the IA-2-134 probe by a standard plaque hybridization procedure (Sambrook *et al.*, 1989). Ten clones were obtained from the primary screen with the insert size ranging from 0.5 to 3.0 kb. A second screen of the same library was performed using a 5′ region probe (713 bp) which was generated by *Bst* XI restriction enzyme digestion of IA-2-4. Thirteen additional cDNA clones were obtained that contained sequences overlapping with the 5′ region of the IA-2 molecule.

Sequencing

Plasmid DNAs from various cDNA clones were isolated using the Qiagen plasmid kit (Chatsworth, CA) and used for double-stranded DNA sequencing. DNA sequencing was performed using Sequenase T4 DNA polymerase under conditions recommended by the supplier (U.S. Biochemical Corp., Cleveland, OH). Internal sense and antisense strand primers were synthesized by Bio-Synthesis, Inc. (Denton, TX). The full-length cDNA nucleotide sequence was derived by sequencing 24 independent overlapping clones obtained from both the subtraction library and random-primed λZAPII library. DNA sequences were analyzed using a Model VAX 750 (Digital Electronics Corporation computer) and GCG Sequence Analysis software package (Devereux *et al.*, 1984). The current FASTA database was used for searching both nucleic acid and protein sequence similarities (Pearson and Lipman, 1988).

In vitro *translation of IA-2 transcript*

A cDNA clone containing the complete IA-2 open reading frame was constructed from overlapping clones, IA-2-4 and IA-2-3, by splicing at the unique restriction site, *Afl* III (Fig. 1A). Capped mRNA (Stratagene) was synthesized using either T3 or T7 RNA polymerase to produce both the sense and antisense transcripts. One microgram of transcript was added to a rabbit reticulocyte *in vitro* translation reaction mixture (Promega) in the presence of [^{35}S]cysteine (Amersham) at 30°C for 1 hr. Twenty-five microliters of reaction mixture was run on a 12% $NaDodSO_4$-PAGE and then fixed with autoradiography enhancer (NEN, Boston, MA). The gel was exposed to film for 12 hr.

Northern analysis

Northern analysis was performed using total cellular RNA isolated by the acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). RNA samples (20 μg each or stated otherwise) were fractionated on a 1% agarose/formaldehyde gel and transferred onto Nytran membrane (Schleicher & Schuell, Keene, NY) *via* capillary blotting. The quality and quantity of electrophoresed RNAs were verified by 18S and 28S ribosomal RNAs. Hybridization was performed at 50°C for 18 hr in a solution containing 40% formamide, 5× SSC, 10 μg/ml sheared salmon sperm DNA, 6× Denhardt's solution, and 10^6 cpm/ml ^{32}P-labeled probe. Northern blots were exposed either overnight or for 3–4 days. The cDNA insert was excised from plasmid vector and purified by the Geneclean II kit (Bio 101, Inc., La Jolla, CA). Two hundred nanograms of the purified insert was labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL), using a commercially available random-primed labeling kit (BRL, Bethesda, MD) and purified by Nick column (Pharmacia, Piscataway, NJ).

Isolation of mouse islets

Pancreatic islets from female BALB/c mice were enriched as described by Brunstedt *et al.* (1985). Briefly, pancreas from 20 mice were digested with collagenase P (Boehringer Mannheim, Indianapolis, IN) and islets were isolated by Percoll gradient (Phamacia, Uppsala, Sweden) separation. The enriched islets then were extracted for total RNA. Insulin message was detected with a rat insulin probe (Lomedico *et al.*, 1979).

RESULTS

Isolation and expression of IA-2 cDNA clones

A cDNA library of 153 clones (ISL-153) was constructed by subtraction of a human glucagonoma phagemid library from a human insulinoma phagemid library (Goto *et al.*, 1992). Several clones were identified by differential screening with ^{32}P-end-labeled mRNA probes derived from insulinoma, glucagonoma, and HeLa cells. Each clone was sequenced (approximately 200 bp) and compared with

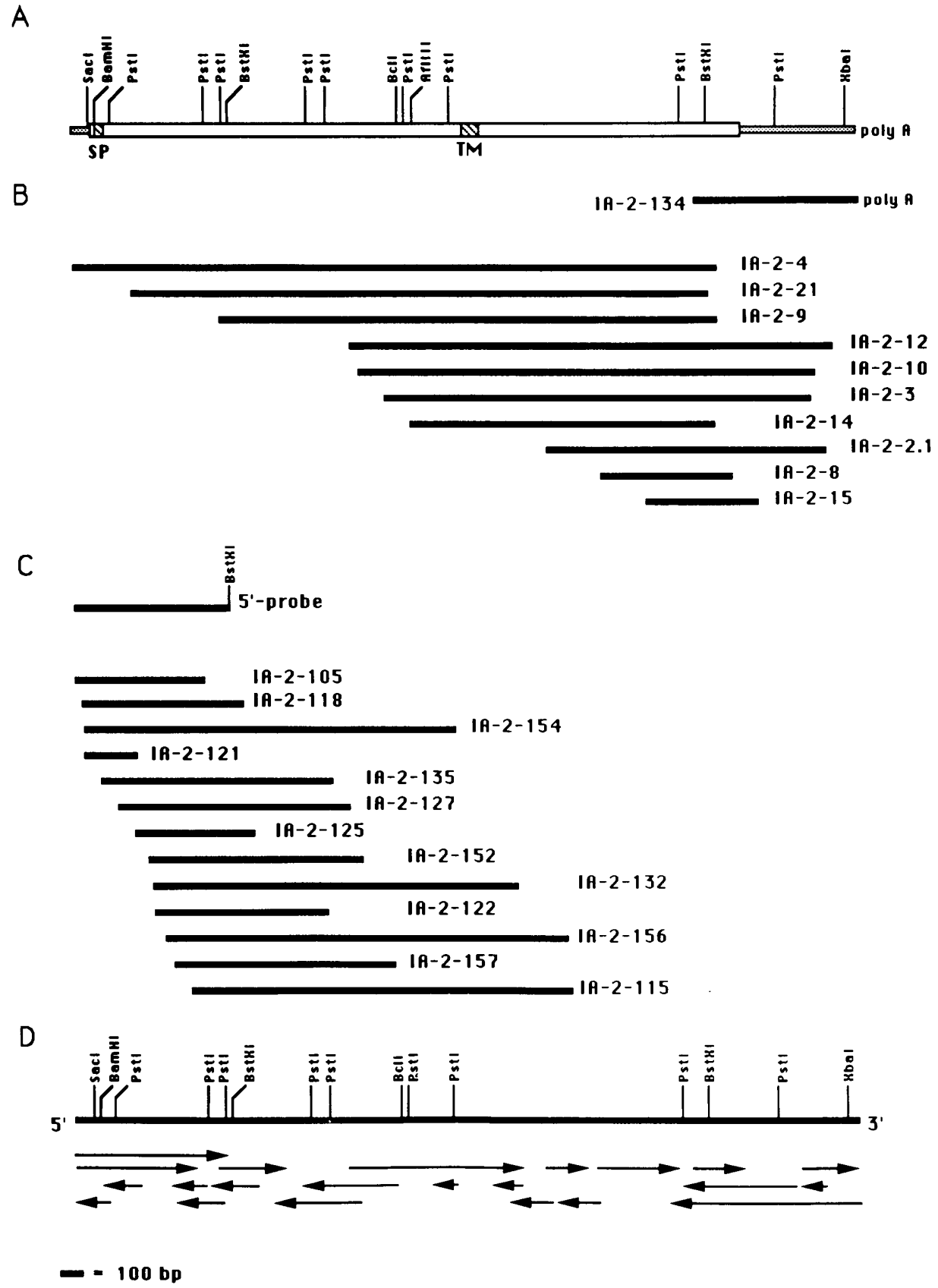
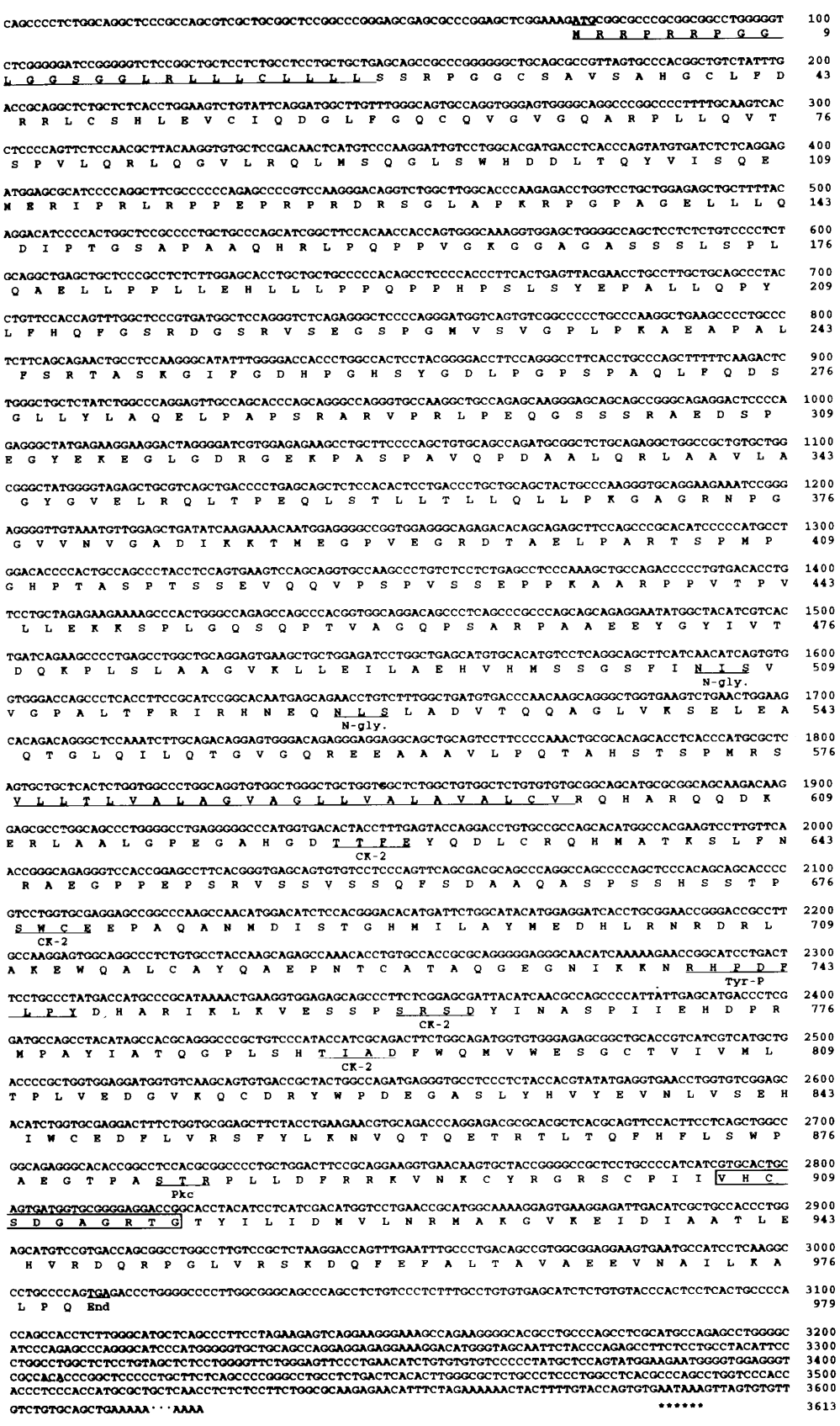


FIG. 1. IA-2 cDNA clones. A. Full-length IA-2 cDNA. Restriction map of the full-length IA-2 sequence is shown at the top of the figure. Open box, open reading frame; SP, signal peptide (amino acids 1–25); TM, transmembrane domain (amino acids 577–600). B. IA-2-134 clone was isolated from the primary subtraction library and extended 749 bp from the poly(A) tail. Ten clones were isolated from a random-primed human λZAPII insulinoma library by using IA-2-134 as a probe. C. A 5′ probe was generated by *Bst* XI restriction enzyme digestion of IA-2-4. Thirteen additional clones were obtained from rescreening the same random-primed human λZAPII insulinoma library with the 5′ probe. D. Sequencing strategy of IA-2 cDNA. The nucleotide sequence was derived from different cDNA clones using specific oligonucleotide primers. Lengths and orientations of the sequence regions are indicated by the arrows.

A



known sequences in the GenBank DNA database as described previously. A unique clone, IA-2-134, which hybridized differentially with human insulinoma and glucagonoma mRNAs, but not with HeLa cell mRNAs, was selected for further study. Analysis of IA-2-134 revealed a sequence of 749 bp with a poly(A) tail (Fig. 1B). To obtain the full-length sequence (Fig. 1A), a random-primed λZAPII cDNA library (RP-IL) from human insulinoma was constructed and screened with IA-2-134 cDNA probe. Ten clones with sizes ranging from 0.5 to 3.0 kb were obtained from the primary screen (Fig. 1B). The longest clone, IA-2-4, was 2,959 bp in length and overlapped with IA-2-134. Together, IA-2-4 and IA-2-134 totaled 3,607 bp, not including the poly(A) tail. To isolate cDNA clones extending further upstream of IA-2-4, a 5′-region probe (*i.e.*, a *Bst* XI restriction enzyme digestion of IA-2-4, Fig. 1C) was used to rescreen the same library. An additional 13 cDNA clones were obtained that overlapped with the 5′ region of IA-2-4 (Fig. 1C). One of these clones, IA-2-105, was found to have six additional base pairs upstream of IA-2-4. cDNA clones of various lengths were subjected to double-strand sequencing by using internal primers from both directions. The complete nucleotide sequence of IA-2 cDNA, shown in Fig. 2A, was determined according to the strategy depicted in Fig. 1D.

We subcloned the complete IA-2 cDNA by ligating two overlapping clones. Sense RNA transcript was synthesized *in vitro* from T3 polymerase promoter. Using the rabbit reticulocyte lysate *in vitro* translation sytstem, a prominent protein product with an estimated M_r of 106,000 daltons was produced (Fig. 3A). The molecular mass of translated protein agrees closely with that predicted from the first start codon (position 74) of IA-2 cDNA sequence. Antisense transcript (Fig. 3B) revealed no apparent protein product.

IA-2 nucleotide and protein sequence

The full-length IA-2 cDNA, derived from 24 clones, consists of 3,613 bp. The cDNA sequence (Fig. 2A) represents a 73-bp 5′ untranslated region and a 600-bp 3′ untranslated region with a polyadenylation signal, AATAAA, at position 3,584. There are several start codons (ATG) in the 5′ region, each constituting an open reading frame. The ATG at position 74 was selected as the start codon because it is the longest open reading frame that would encode a hydrophobic signal peptide. In the *in vitro* translation system, the size of translated protein revealed that the first ATG initiation codon is the dominant start site of IA-2 cDNA. The deduced protein is 979-amino-acid-long with a molecular mass of 105,847 daltons. A hydrophobicity plot of the deduced amino acid sequence reveals three major hydrophobic areas (Fig. 2B). On the basis of the criteria of both Kyte and Doolittle (1982) and Klein *et al.* (1985), stretches of hydrophobic amino acids at positions 1–25 and 577–600 represent a putative signal peptide and a transmembrane region, respectively. Residue 801–813 displays sufficient hydrophobicity, but contains less than the minimal number of amino acids (*i.e.*, amino acid 17), to represent a membrane-spanning segment. The extracellular domain of IA-2 (amino acids 26–576) has 550 amino acids containing an unusual cysteine-rich region (>15%) located next to the signal peptide. In addition, the sequence of IA-2 revealed two extracellular N-linked gly-

B

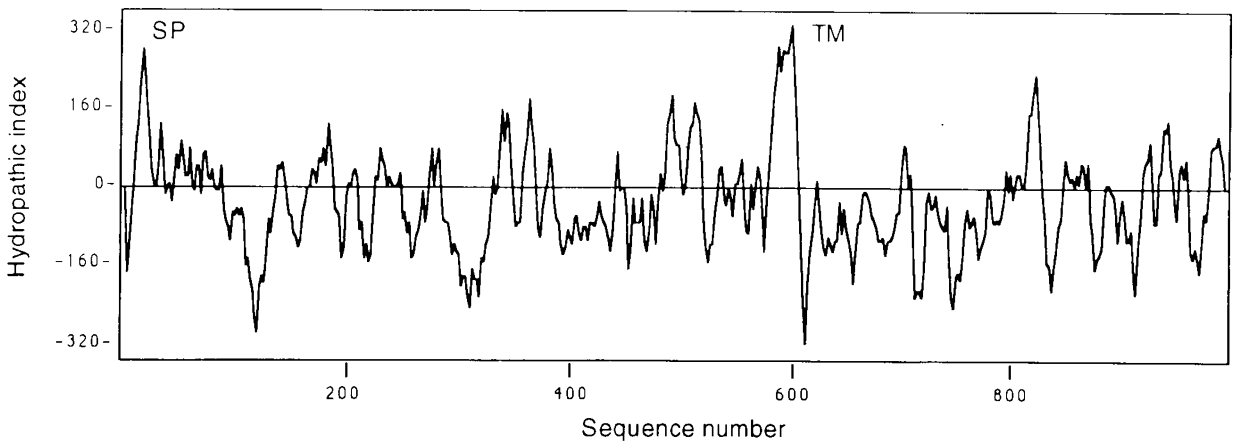


FIG. 2. Nucleotide and deduced amino acid sequence of a human insulinoma-associated cDNA, IA-2. A. Nucleotide and protein sequence. The deduced translational start codon, ATG, is designated as position 1 and the stop codon, TGA, as position 980. The segments underlined indicate a putative signal peptide sequence (amino acids 1–25) and transmembrane region (amino acids 577–600); Pkc, putative protein kinase C phosphorylation site; Tyr-P, putative tyrosine phosphorylation site; N-gly, putative N-linked glycosylation site. An 11-amino-acid "core sequence" of protein tyrosine phosphatase is boxed. The asterisk (*) indicates a polyadenylation signal sequence. B. A hydrophobicity profile of the deduced protein is plotted according to Kyte and Doolittle (1982).

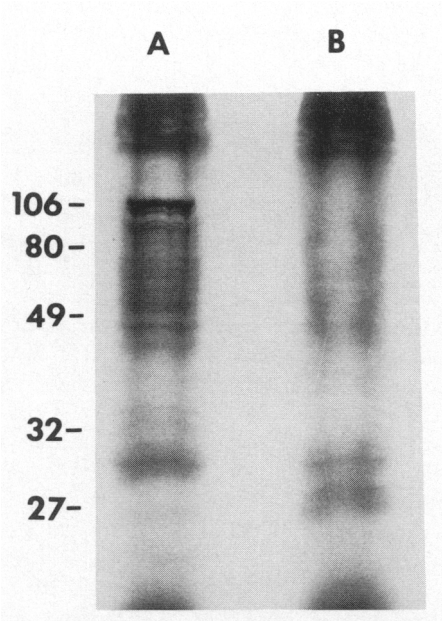


FIG. 3. *In vitro* translation of IA-2 mRNA transcripts. Both sense (A) and antisense (B) RNA transcripts were translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]cysteine and analyzed on a 12% NaDodSO$_4$-PAGE as described. Molecular mass markers ($M_r \times 10^{-3}$) are shown at the side.

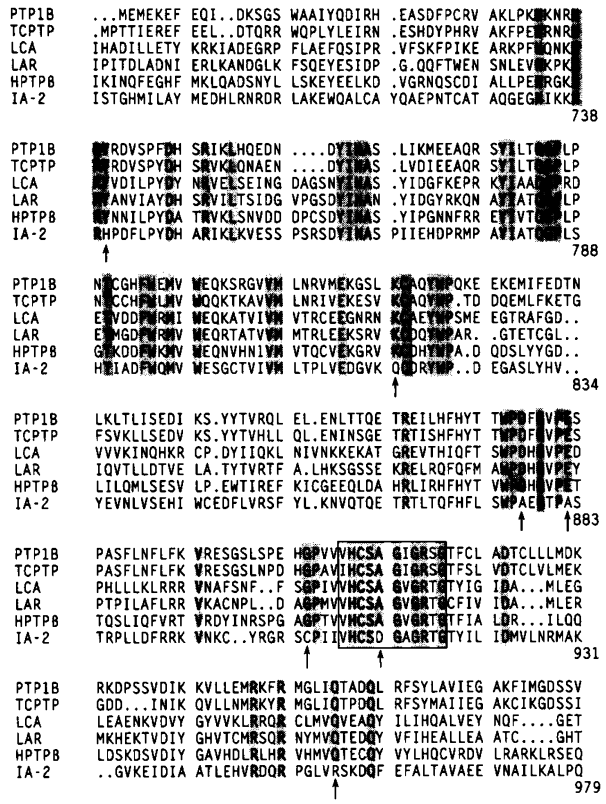


FIG. 4. Amino acid sequence alignment of conserved domains from proteins of the PTP family: PTP1B, human placenta (Chernoff *et al.*, 1990); TCPTP, T-cell PTP (Cool *et al.*, 1989); LCA, leukocyte common antigen (CD45), first domain (Ralph *et al.*, 1987); LAR, leukocyte antigen-related PTP, first domain (Streuli *et al.*, 1988); HPTPβ, human PTPβ (Krueger *et al.*, 1990) and IA-2. Residue numbers are those of IA-2. Shaded areas identify residues that are identical in all the PTP sequences. The core sequence of PTP is boxed. Arrows indicate change in charge or hydrophobicity of IA-2 amino acid in highly conserved regions.

cosylation sites (Asn-X-Ser/Thr) and intracellular Ser/Thr-phosphorylation and Tyr-phosphorylation sites (Fig. 2A).

The intracellular domain (amino acids 701–979) of IA-2 is of particular interest because it bears similarity with the catalytic domain of members of the PTP family (Charbonneau *et al.*, 1989). The catalytic domains consist of a "core sequence," 11 amino acids long, (V/I)HCSAG(V/I)-GR-(T/S)G (Streuli *et al.*, 1990), with an essential cysteine residue and a GXGXXG nucleotide binding motif (Taylor *et al.*, 1990). The core sequence of IA-2 is found at position 907–917 (Fig. 2A). Five representative human PTPs and the cytoplasmic domain of IA-2 are compared in Fig. 4 using the multiple sequence alignment method of Feng and Doolittle (1987). Conserved amino acids are distributed in a region of approximately 300 residues surrounding the "core sequence." Figure 4 shows substitutions in the conserved regions of the IA-2 molecule.

Tissue expression of IA-2 gene

Human tissues were examined for IA-2 message by Northern analysis using ^{32}P-labeled IA-2-134 and IA-2-4 as the probe. Figure 5A shows that a 3.8-kb mRNA was detected strongly in four of five human insulinomas, weakly in one insulinoma and one glucagonoma. Figure 5B shows that IA-2 was expressed in normal human brain, pituitary, and 40 μg of pancreas RNAs. Figure 5C also shows a strong signal in the glioblastoma cell line (U-87-MG), whereas neuroblastoma (SK-N-SH) and thyroid carcinoma (SW579) cell lines are weakly positive after prolonged exposure, which is not shown here. IA-2 mRNA was not detected in other normal tissues, including lung, lymph node, thyroid, testes, liver, colon, kidney, stomach, small intestine, and spleen. A variety of other cell lines, such as choriocarcinoma (JAR), breast carcinoma (BT-20), melanoma (DM-6, SKMEL), pancreatic carcinoma (HPAF-2), colon carcinoma (LS-180), prostate carcinoma (PC-3), and HeLa cells also were negative by Northern analysis.

Murine tissues also were examined for IA-2 expression by Northern analysis. Figure 6 shows that IA-2 was expressed in both mouse and rat insulinoma cell lines. It also was detected in enriched normal mouse islets, but not whole pancreas. The absence of detectable IA-2 mRNA in whole pancreas is consistent with the small proportion of islet cells (less trhan 2%) in the tissue (Gepts and LeCompte, 1985).

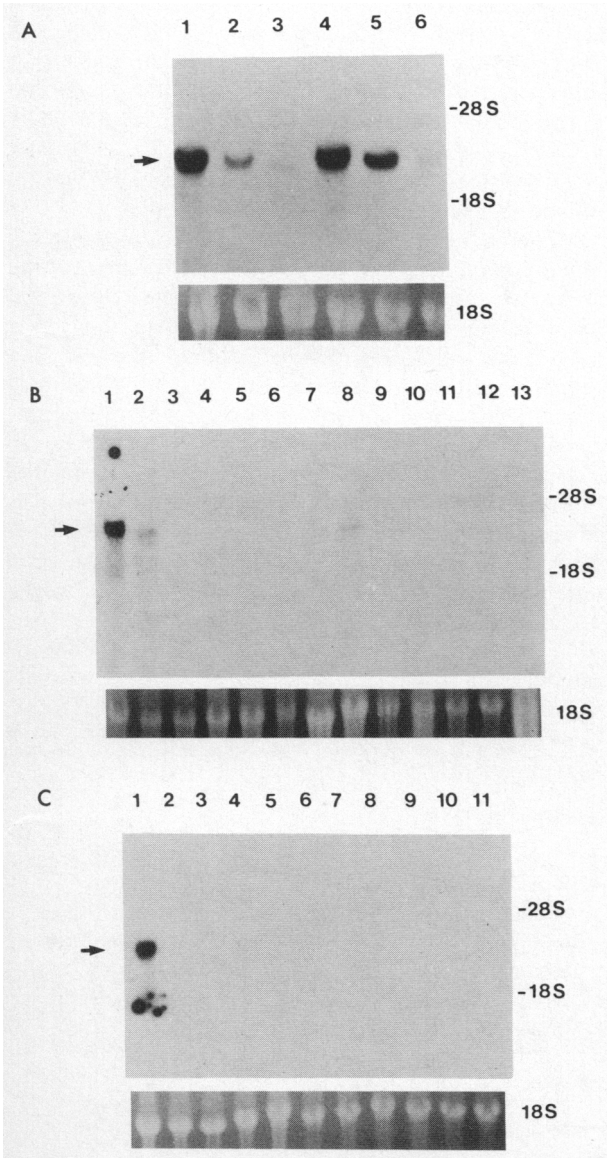


FIG. 5. Northern analysis. Total RNA isolated from human insulinoma and glucagonoma tumor tissues (A), normal human tissues (B), and tumor cell lines (C) were separated on a 1% agarose/formaldehyde gel and hybridized with ^{32}P-labeled IA-2 cDNA probe. Twenty micrograms of RNA were used in each lane unless indicated elsewhere. A. Insulinomas and glucagonoma: five different human insulinomas (lanes 1–5) and a human glucagonoma (lane 6). B. Normal tissues: brain (lane 1); pituitary (lane 2); lymph node (lane 3); thyroid (lane 4); thymus (lane 5); lung (lane 6); liver (lane 7); 40 μg of pancreas (lane 8); stomach (lane 9); colon (lane 10); kidney (lane 11); testes (lane 12); and spleen (lane 13). C. Human tumor cell lines: glioblastoma, U-87-MG (lane 1); neuroblastoma, SK-N-SH (lane 2); choriocarcinoma, JAR (lane 3); thyroid carcinoma, SW579 (lane 4); breast carcinoma, BT-20 (lane 5); melanoma, DM-6, SKMEL (lanes 6 and 7); pancreatic carcinoma, HPAF-2 (lane 8); colon carcinoma, LS-180 (lane 9); HeLa (lane 10); and prostate carcinoma, PC-3 (lane 11). The filter was exposed for 3 days. Message size of IA-2, 3.8 kb (arrow) was estimated by ribosomal RNAs. 18S ribosomal RNAs are shown at the bottom of blots.

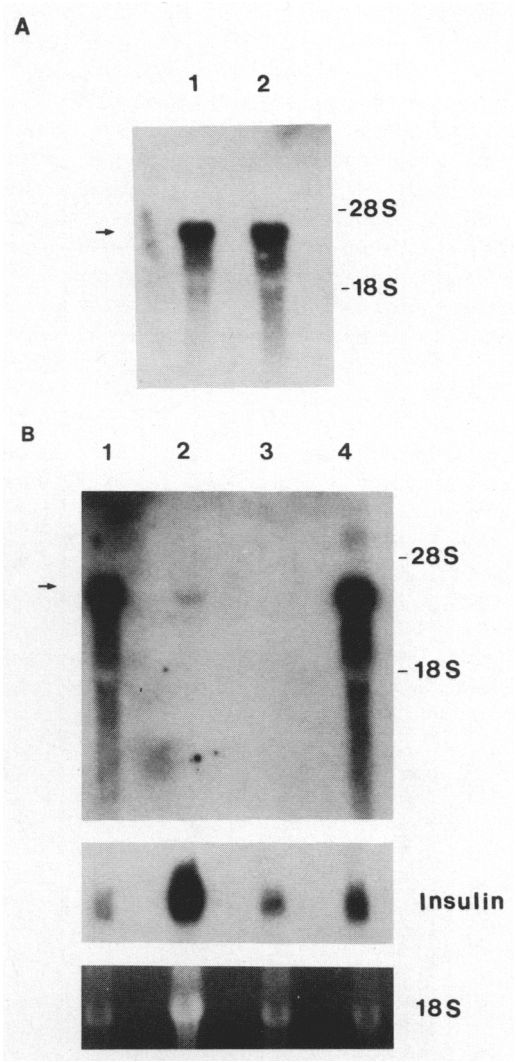


FIG. 6. Northern analysis. Total cellular RNAs isolated from whole mouse pancreas, enriched mouse islets, and murine insulinoma cell lines were separated on a 1% agarose/formaldehyde gel and hybridized with ^{32}P-labeled IA-2-4. A. Lane 1, 20 μg of mouse insulinoma, βTC-1; lane 2, 20 μg of rat insulinoma, RIN. B. Lane 1, 5 μg of βTC-1; lane 2, 20 μg of enriched mouse islets; lane 3, 20 μg of whole mouse pancreas; lane 4, 10 μg of βTC-1. Filter exposed for 16 hr (A) or 96 hr (B). The message size of IA-2, 3.8 kb (arrow), was estimated by ribosomal RNAs. 18S ribosomal RNAs are shown at the bottom of the blot. Increased insulin message detected in the enriched islets (lane 2) by hybridization with an insulin probe.

DISCUSSION

From a human insulinoma cDNA subtraction library, we isolated a novel cDNA, IA-2. Analysis of IA-2 sequence and *in vitro* translation data revealed a full-length nucleotide sequence of 3,613 bp and a deduced protein of 979 amino acids with features consistent with a transmembrane protein.

A GenBank database search showed sequence similarity between the intracellular domain of IA-2 and representa-

tive members of the PTP family (Ralph *et al.*, 1987; Cool *et al.*, 1989; Streuli *et al.*, 1988; Chernoff *et al.*, 1990; Krueger *et al.*, 1990). PTPs are enzymes that catalyze the removal of a phosphate group attached to a tyrosine residue by protein tyrosine kinase (PTK). Tyrosine phosphorylation and dephosphorylation have been recognized as important steps in regulating cellular processes, including growth, differentiation, and transformation. Alignment of IA-2 cytoplasmic domain with five representative human PTP domains demonstrated multiple highly conserved regions along a stretch of approximately 300 amino acids. Clusters of conserved regions surrounding the core sequence are found in most, if not all, PTP molecules (Krueger *et al.*, 1990).

The intracellular segment of IA-2 contains only a single PTP domain that distinguishes it from most of the other transmembrane receptor-like PTPs that contain two tandem copies of the PTP domain, as shown in Fig. 7 (Streuli *et al.*, 1988; Krueger *et al.*, 1990; Gebbink *et al.*, 1991, 1993; Trowbridge *et al.*, 1991; Krueger and Saito, 1992; Barnea *et al.*, 1993; Levy *et al.*, 1993). In this way, IA-2 is similar to HPTPβ (Krueger *et al.*, 1990). The extracellular domain of IA-2 (576 amino acids) contains an unusual cysteine-rich region following a signal peptide. The presence of this cysteine-rich region raises the possibility of secondary structure that might serve as a ligand binding site (Durkop *et al.*, 1992). The extracellular domain of IA-2, in contrast to the extracellular domains of HPTPβ, HPTPδ (Krueger *et al.*, 1990), LAR (Streuli *et al.*, 1988), HPTPμ (Gebbink *et al.*, 1991), DLAR (Streuli *et al.*, 1989), and DPTP (Hariharan *et al.*, 1991), does not contain fibronectin type III-like or immunoglobulin-like regions.

By comparing the "core sequence" of IA-2, VHCSDGAGRTG (amino acids 907–917) with other members of the family, two substitutions were found in this region. The first substitution, at position 911 (Ala to Asp), changes a hydrophobic amino acid to a hydrophilic amino acid. The second substitution, at position 913, changes Val to Ala. In addition, several substitutions were found in the conserved regions outside the core sequence of IA-2 (*i.e.*,, at positions 740, 819, 877, 882, 903, and 954). Changes in the core region can decrease or abolish PTP activity when tested with standard substrates (Streuli *et al.*, 1990). Currently, we are attempting to express and determine the substrate specificity of IA-2 and the role that this PTP might play in signal transduction.

The demonstration that IA-2 mRNA is expressed only in islet and brain tissues suggests that IA-2 might be a tissue-

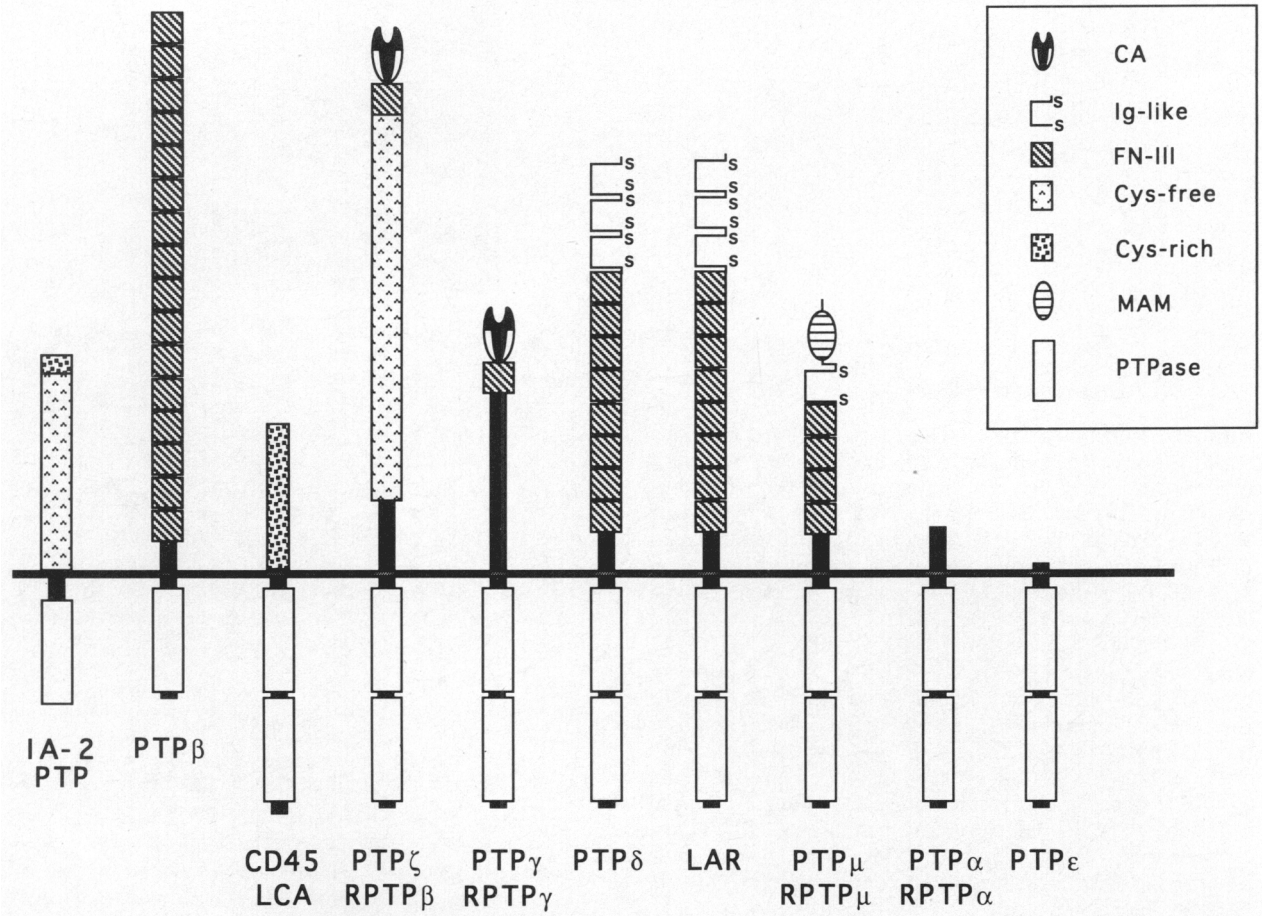


FIG. 7. Human transmembrane receptor-type protein tyrosine phosphatase. The carbonic anhydrase (CA), immunoglobulin (Ig)-like, fibronectin type III (FNIII), cysteine (cys)-free, cysteine (cys)-rich, Meprin, A5, μ (MAM), and PTPase domains are represented schematically.

restricted PTP. Examples of tissue-restricted PTPs include CD45, a leukocyte PTP, expressed exclusively in hematopoietic cells (Trowbridge *et al.*, 1991); STEP, a striatum-enriched phosphatase, expressed in neural tissue (Lombroso *et al.*, 1991); HPTPζ, expressed in brain (Krueger and Saito, 1992; Levy *et al.*, 1993); and two *Drosophila* receptor-like PTP genes, DPTP99A and DPTP10D, expressed primarily in the central nervous system (Yang *et al.*, 1991). In this connection, it is of interest that pancreatic islet cells express a large number of neuroendocrine markers (Alpert *et al.*, 1988; Teitelman, 1990) and that the γ-aminobutyric acid (GABA)-synthesizing enzyme, glutamic acid decarboxylase (GAD), a 64-kD autoantigen thought to be important in IDDM, is found in both the brain and islets (Solimena *et al.*, 1990). Patients with IDDM have autoantibodies to this antigen (Christie *et al.*, 1988; De Aizpurua *et al.*, 1992; Rowley *et al.*, 1992). The possibility that patients with IDDM also may have autoantibodies to IA-2 will be investigated when sufficient amounts of this protein are produced.

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The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number L18983.

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